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Microbial Diversity Associated With Rice Seeds

MUHAMMAD ASHFAQ[*1], QURAT-UL-AIN ASGHAR[1], RAHILA HAFEEZ[1], AMNA ALI[1], MUHAMMAD SALEEM HAIDER[1], MUHAMMAD ALI[1], MUHAMMAD BILAL CHATTHA[1] ABDUL RASHEED[1] & MUHAMMAD SAJJAD[2]

[1]Institute of Agricultural Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
[2] Environmental Sciences COMSATS Institute of Information Technology, Pakistan

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***Corresponding Author:**

Muhammad Ashfaq
Email: ashfaq.iags@pu.edu.pk

ABSTRACT

During present investigation, seed-born micro-flora of five rice (*Oryza sativa* L.) varieties viz. Basmati-198, Basmati-802, Basmati Pak, Basmati Shaheen and Basmati-370 were studied using agar plate and blotter paper method. Highest percentage of myco-flora (17%) was found to be associated with seeds of Basmati Pak that was considerably more than myco-flora associated with all other varieties of rice. The highest percentage of bacterial isolates (16%) was recorded with Basmati Shaheen variety. Total of six fungal and three bacterial species namely *Fusarium* sp., *Alternaria alternata*, *Pyricularia* sp., *Dreschslera* sp., *Penicillium* sp., *Curvularia* sp*., Acetobacterium, Deniobacter* and *Micrococcus* were isolated from different rice varieties.

Key Words: Rice, seed-borne micro-flora, blotter paper, diversity

INTRODUCTION

Oryza sativa (rice) is a member of the grass family (Arnold *et al.,* 1987). As a cereals crop, it is generally consumed as food for most of the world's population, especially in Asia (Neue, 1993). Cereals manufacturing in Pakistan keeps very important position in farming and the national economic system (Abid *et al*., 2014). Pakistan is the world's 11th biggest manufacturer of rice, after China, India, Indonesia, Bangladesh, Vietnam, Thailand and Burma. Each year, it produces an average of 6 million tones and together with the rest of the South Asia, the country is responsible for providing 30% of the paddy grain output (FAO, 2006). Many fungi, ranging from pathogens to non-pathogens of rice, have been recorded as seed borne on rice (Aluko *et al.,* 2004). The rice crop has suffered from various types of diseases, majority of them are known to be caused by fungi e.g sheath spot (*Cerato basidium*) Black kernel (*Curvularia lunata*, *Cochliobolus lunatus*) Blast (leaf, neck and collar) (*Pyricularia grisea*, *Pyricularia oryzae*), kernel spotting (*Curvularia*, *Fusarium*) Root rots (*Fusarium* , *Pythium*) and seedling blight (*Curvularia*, *Fusarium*, *Rhizoctonia solani*) (Dean *et al*., 2005). Rice seed is affected by wide range of bacteria. The rice crop has been suffered from various type of diseases, majority of them are known to be caused by bacteria e.g Bacterial blight (*Xanthomonas oryzae*) Bacterial leaf streak (*Xanthomonas oryzae*) Foot rot (*Erwinia chrysanthemi)* Grain rot (*Burkholderia glumae*) Sheath brown rot (*Pseudomonas fuscovaginae*) (Dean *et al.*, 2005). The current study was conducted to recognize the fungal and bacterial micro-flora related to different varieties of rice seeds.

MATERIAL AND METHODS

Sample Collection

Seeds of following rice verities namely Basmati-198, Basmati-802, Basmati Pak, Basmati Shaheen, Basmati-370 were collected from Rice Research Institute of Kala Shaha Kaku, Pakistan. One thousand seed of each variety was selected randomly. Physical characteristics of all the seed varieties were measured by means of seed length, seed width, seed thickness, seed length width ratio and weight.

Selection of Seeds

Seeds from each rice samples were randomly selected and tested for different physical traits (seed length, seed width, seed thickness, length/width, and 1000 seed weight) and germination tests.

Author's contribution: M.A., Supervise the work, helped n planning writing the manuscript; **Q.U.A.A.,** Data Collection and conduct the experiment; **R.H., A.A.,** Helped in data analysis; **M.S.H.,** Helped in reviewed and writing; **M.A.,** Helped in data collection & writing; **M.B.C.,** Recording observation; **A.R.,** Look after the experiment; **M.S.,** Overall reviewed the article

Measurement of Physical Characteristics of Local Germplasm lines

Physical characteristic of 5 local varieties of rice were measured. For this purpose, 1000 seed weight for each sample was measured on a digital weighing balance, and three readings were taken for each sample. Seed length, seed width and seed thickness of these local rice varieties was measured with the help of a digital Vernier Calliper. For each sample of rice, these measurements were taken in three replicates; in each replicate 5 seeds were measured. The seeds were randomly selected from the seed samples.

Selective isolation procedures and media

Rice seeds were air dried for 48 hours at room temperature and then thoroughly washed to remove surface debris completely. After drying, the seed samples were divided in to two groups i.e. unsterilized and sterilized. The seed samples were subjected to surface sterilization procedure: 2-3 min wash in 1% NaOCl, followed by a 5 min wash in sterile water. After being thoroughly dried under sterile conditions, the seeds were then pretreated by one of the following methods for isolation purposes.

Method 1

Seeds samples from both groups were aseptically placed on selective medium i.e. MEA (Malt Extract Agar) and LBA (Laura Bertani Agar) for fungal and bacterial isolation. The inoculated plates were incubated at 25±2 ºC for 5-7 days and at 37 ºC for 24 hours respectively.

Method 2

Occurrence of seed borne micro-flora on seeds was determined by Modified Blotter paper method. Ten seeds of each cultivar were selected. Ten surface sterilized and unsterilized seeds of each cultivar were spaced on Blotter paper in plastic petri dishes. These plastic petri dishes were incubated at -20 ºC for 8 days for breaking seed dormancy. The infested seeds were transferred to MEA and LBA medium plates. The inoculated plates were incubated at 25±2 ºC for 5- 7 days and at 37 ºC for 24 hours respectively. The isolated colonies were subsequently sub cultured in order to obtain pure cultures. The percentage frequency of various fungal and bacterial varieties was calculated as follows:

$$\text{Frequency of occurrence (\%)} = \frac{\text{No. of seeds on which a microbial species occurs}}{\text{Total No. of seeds}} \times 100$$

Identification of Fungal Isolates

The isolated pure fungal colonies were subjected to macro and microscopic identification.

i. Colonial morphology

Pure and fresh fungal isolates were identified using cultural and morphological features such as; colony growth pattern, color, texture and growth elevation. From the incubated plates different fungal isolates with different coloration included; (a) White (b) Brown (c) Grey black, which signified the %age occurrence of different fungal colonies (Emory, 2007; Mirza *et al*., 1979).

ii. Cellular morphology

A small piece of mycelia from representative culture was placed on the glass slide containing lecto-phenol cotton blue stain for colored cultures and trypan blue for white culture colonies using a sterile inoculating needle. The morphological characteristics and appearance of various fungal isolates from rice seeds were confirmed and authenticated with the help of Identification manual (Emory, 2007; Mirza *et al*., 1979).

Biochemical Test and Identification of Bacterial Isolates

For bacterial isolation the surface sterilized sample was cut in to small portions and placed on LBA (Luria Bertani Agar) media plates under aseptic conditions. Inoculated plates were incubated at 37 ºC for 24hours. Different bacterial isolates were observed from inoculated plates, which signified the %age occurrence of various bacterial isolates. Bacterial isolates were re-cultured in order to obtain pure bacterial isolates. Identification of bacteria was carried out following the standardized procedure starting with the colony and cell morphology followed by Gram staining and finally testing the metabolic activities of unknown strain. Identification of bacterial species was done by recording colony morphological features (Beishir, 1991) (color, shape, size, texture, margins and odor etc.) and cell microscopic characters (color, cell wall, contents,

shape, arrangement, material, Gram stain, spore stain, capsular material and motility). The pure colonies were differentiated by biochemical tests (Holt *et al*., 1997; Benson, 1996).

Statistical analysis:

All the collected data was evaluated by Analysis of variance followed by Duncan's Multiple Range Test to separate the treatment means at P≤0.05 (Steel *et al*., 1997).

RESULTS AND DISCUSSION

In present study various physical parameters of different rice varieties were studied. Results exhibited the Basmati-198 (10.36mm) had the healthiest looking grain with husk among fine and coarse rice varieties used for study; since it had longest grain, equal in seed width and thickness with Basmati-Shaheen (2.93mm and 2.92mm). Among fine grain, Basmati-198 had highest 1000 seed weight (30.2g) whereas Basmati-370 had the least weight of 17.2g (Table I & II). Analysis of variance indicated different effects of genotypes in almost all seed morphological traits at various levels of significance (P<0.01 and 0.05). All the genotypes showed significant differences among all the traits studied. Highly significant variations associated with high genetic diversity among the genotypes in all the desirable traits studied are helpful for further screening and selection process. Variance analysis also indicated polymorphism of the genotypes with respect to their various morphological traits (Ashfaq *et al.,* 2013). These will also be providing the basic information to the scientific community, researcher and farmers community (Li *et al*., 2008). Pearson correlation analysis showed significant positive, significant negative and non-significant correlation in some seed morphological traits. Most of the traits showed positive association with each other and considering important morphological tool for the selection of high yielding diverse genotypes (Muthuramu *et al.,* 2010). 1000 grain weight showed highly significant correlation with seed length, seed thickness and seed length width ratio (r= 0.6767**, r= 0.4617**, r= 0.4145**) and significant association with seed width (r= 0.4699*). On the other hand, seed length width ratio showed positive significant association with seed width and seed thickness (r= 0.4615*, r= 0.4692*) and seed width associated width seed length (r= 0.7135*) (Simple correlation of all these seed morphological and root shoot traits are shown in the table II. Various fungal and bacterial species were isolated from different rice varieties. The morphological data is shown in tables III & IV. Highest percentage of myco-flora (17%) was found to be associated with seeds of Basmati-Pak that was considerably more than myco-flora associated with all other seed types. While highest percentage of bacterial isolates (16%) was recorded with Basmati-Shaheen variety. There was 14%, 13%, 12% and 10% bacterial isolates associated with the seeds of Basmati-370, Basmati-198 and Basmati- 386, respectively. In figure 1, *Fusarium* sp., *Alternaria alternata*, *Pyricularia* sp., *Dreschslera* sp., *Penicillium* sp. and *Curvularia* sp. were recorded from various rice varieties with varying percentage incidence. Early research has recorded *Alternaria alternata, Aspergillus niger, Curvularia lunata, Curvularia oryzae, Pyricularia oryzae, Drchslera oryzae, Fusarium miniliforme* and *Fusarium oxysporum* from different rice varieties (Khan *et al.,* 2000; Wahid *et al*., 2001; Javaid *et al.,* 2002; Ibiam *et al.,* 2006; Nguefack *et al*., 2008). Greatest percentage of *Alternaria alternata* (16%) was found in seeds of Bas-Pak that was significantly greater than its incidence of all other seed varieties. There was 7%, 9%, 4% and 2% incidence of this fungal species in seeds of Basmati-370, Basmati-386, Basmati- 198 and Basmati- Shaheen, respectively (Figure 2). Distinction in incidence of *Alternaria alternata* among various varieties was insignificant. In the same way, determined percentage of *Fusarium* sp. was documented in seeds of Basmati- 386 (6%) followed by 4.5% in seeds of each of Basmati- 198 and Basmati-370, and 2.5% in seeds of Basmati-Pak and Basmati-Shaheen. Maximum percentage of occurrence of *Curvularia* sp. (3%) was found in Basmati-Pak and Basmati-198 followed by Basmati-386 (2.5%), Basmati-370 (2%) and Basmati-Shaheen (1.5%). Percentage occurrence of *Pyricularia* sp., *Dreschslera* sp. and *Penicillium* sp. was recorded 2.0, 1.0, 2.5, 0.5, 1.0%, respectively in Basmati- 198 and Basmati-370, and Basmati- 386, Basmati-Pak and Basmati-Shaheen. Three bacterial species *Acetobacterium, Deniobacter* and *Micrococcus* were isolated from the various rice varieties under investigation (Ashfaq *et al.,* 2015). Highest

percentage of occurrence of *Acetobacterium* (9%) was recorded in seeds of Basmati-Shaheen followed by 0%, 2% 3.5%, and 4% in Basmati-Pak, Basmati-370, Basmati-386 and Basmati-198, respectively. On the other hand highest percentages of *Deniobacter* and *Micrococcus* (5%, 3%) were found in seeds of Basmati- 370 and Basmati-Shaheen, respectively Fig., 3.

CONCLUSION

Overall, Basmati-198, Basmati-370 and Basmati- Shaheen showed fruitful results that could be used for the screening, assessment and improvement of rice crop for producing healthy rice for nourishing more people. On the other hand, the information obtained from this study may be helpful for breeders, scientists and farmers community for starting a new research program for producing disease free rice in the rice World by utilizing and exploiting the potential rice lines.

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Table I: Analysis of variance of seed morphological traits of various rice genotypes

Source of variation	DF	SL	SW	ST	SL/SW	1000 SW
Genotypes	4	2.05425	0.04096	0.12585	0.00131	0.007
Replications	3	2.31589	0.18038	0.26212	0.90938	228.652
Errors	12	1.95567	0.04814	0.08686	0.02793	0.004

Level of significance p<0.05=* and p<0.01=**
SL= seed length, SW= seed width, ST= seed thickness, L/W=length/width ratio and 1000 grain weight

Table II: Pearson's Correlation among different seed morphological traits of rice

Traits	SL(mm)	SW(mm)	ST(mm)	SL/SW(mm)	1000SW(g)
SL	100				
SW	0.7135	1.00			
ST	0.6829	0.9397	1.00		
SL/SW	0.8287	0.4615	0.4692	1.00	
1000 SW	0.6767	0.4699	0.4617	0.4145	1.00

Level of significance p<0.05=* and p<0.01=**
SL= seed length, SW= seed width, ST= seed thickness, L/W=length/width ratio and 1000 grain weight

Table III: Biochemical characteristics of bacterial isolates encountered

S.No.	Colony characters	Gram stain	Spore	Motility	Biochemical tests								Probable identity of isolates
					Catalase	Citrate Utilization	Urease	Indole	Glucose	Methyl red	Hydrogen Sulfide	Nitrate reduction	
1	Raised smooth spherical entire creamy opaque	+ Rods	-	-	+	-	-	-	-	+	-	-	*Acetobacterium* sp.
2	Raised smooth Spherical entire Translucent	+ Cocci	-	-	-	-	-	-	-	-	-	-	*Deniobacter* sp.
3	Flat smooth spherical entire creamy opaque	+ Cocci	-	-	-	-	-	-	-	-	+	-	*Micrococcus* sp.

Table IV: Microbiological characterization of fungi isolates encountered

S.No.	Colony morphology	Mycelium	Conidial color and shape	No. of septa	Conidial size	Identity of isolates
1	Greenish brown-black, smooth edge, sporulation on surface. Reverse; black	Branch chained, having 4-6 conidia	Brown, ovoid, ellipsoid	Transverse septa; 4-7 Longisepta; 0-2	20-32 x 6-12µm	*Alternaria alternata*
2	White, cottony Reverse; off white	Aerial mycelia, hyaline to colorless	Curved blunt apical, pedicillate basal part	Microconidia: 0-1 septate, blunt Macroconidia: 3-5 septate	25-30x 4-6 µm	*Fusarium* sp.
3	Blackish-brown, velvety Reverse; black	Immersed, dark brown	Ellipsoidal, round at ends, pale brown,	4-5 pseudo-septa	13-30 x 6-10 µm	*Drechslera* sp.
4	Effuse, greyish brown	Pale brown, un-branched,	Solitary, pyriform, olivaceous brown	1-3	17-25 x 6-9 µm	*Pyricularia* sp.
5	Black, velvety Reverse; black	Dark brown, thick walled	Cylindrical, sub-ellipsoidal	4- celled Central 2 cells darker	20-28 x 6.5-8 µm	*Curvularia* sp.
6	Green grey Reverse; off white	Hyaline, erect, penicillated, verticillate phialides	Pale green, sub-globose	No	2-3 µm	*Penicillium* sp.

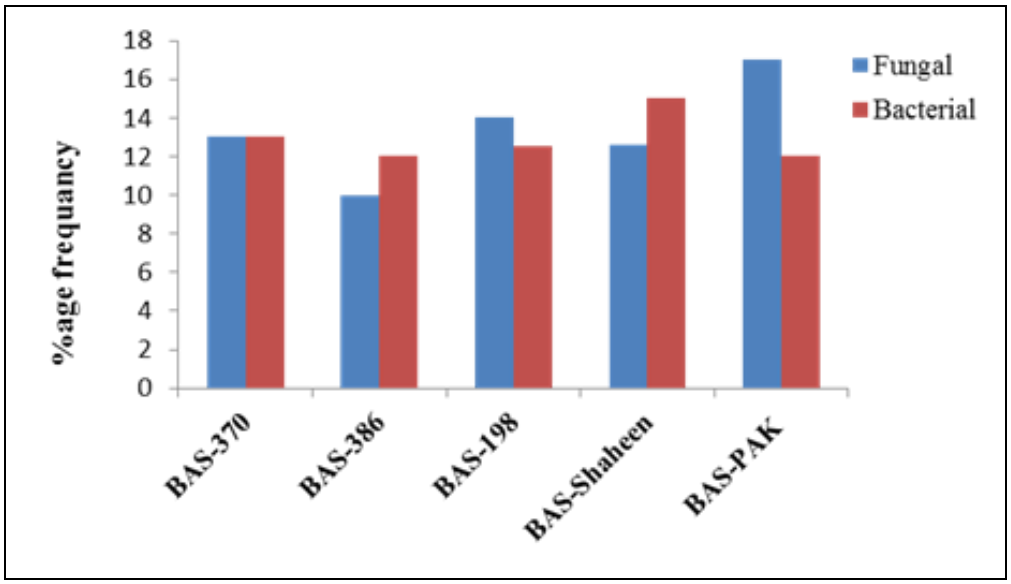

Fig., 1: Percentage of micro-flora associated with different rice varieties

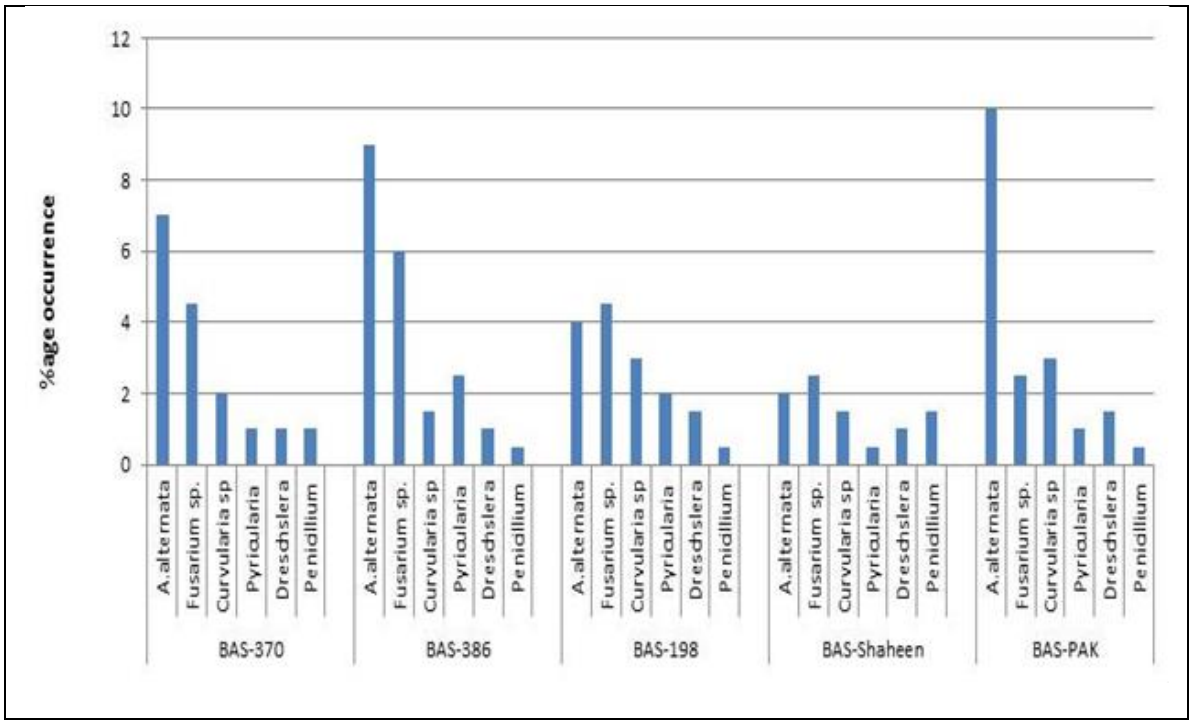

Fig., 2: Percentage occurrence of fungal species in five commonly cultivated rice varieties.

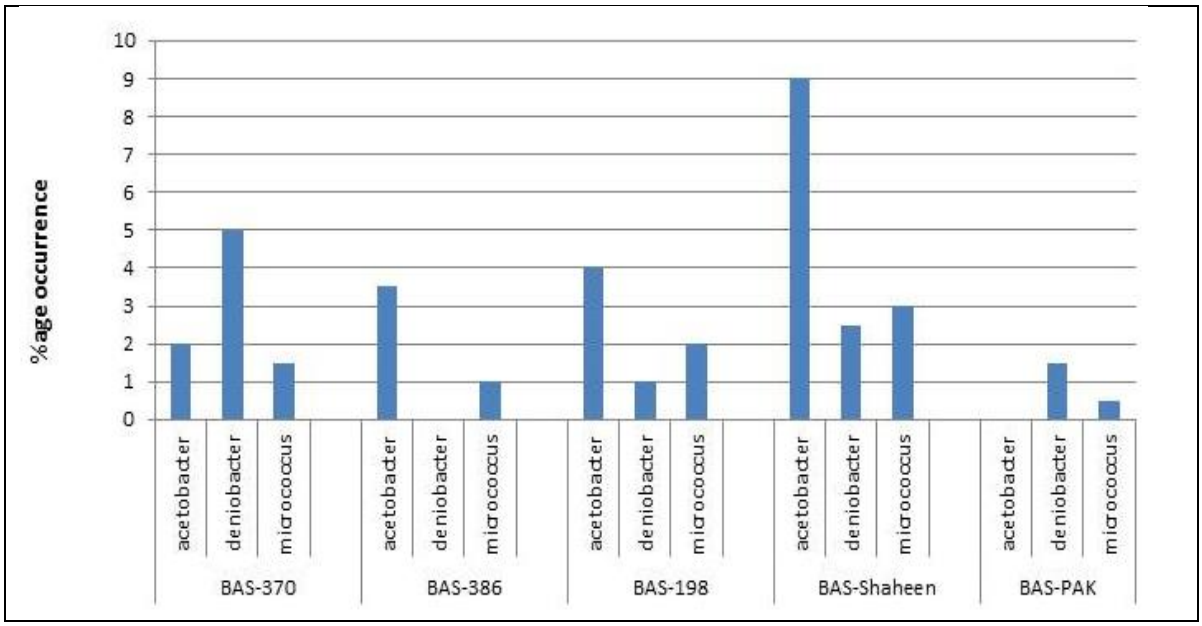

Fig., 3: Percentage occurrence of bacterial species in five commonly cultivated rice varieties.

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